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(54) Title: PROCESS OF DETERMINING THE EFFICACY OF DRUG TREATMENT IN HIV INFECTED SUBJECTS

(57) Abstract

The present invention provides a process for determining the efficacy of anti-viral therapy in an HIV-infected subject receiving such therapy. The process includes the steps of a) detecting the level of transcriptionally active HIV in monocytes of the subject at a plurality of different times, b) comparing the detected HIV levels, and c) correlating changes in the detected HIV levels over time with the therapy. The process can be used to monitor the efficacy of treatment with any anti-HIV agent such as AZT, 3TC, DDc, Indivar, or Saquinavir. Decreases in HIV levels over time indicate an efficacious treatment. Increases in detected HIV levels over time indicate resistance to treatment.

PROCESS OF DETERMINING THE EFFICACY OF DRUG TREATMENT IN HIV INFECTED SUBJECTS

Cross-Reference to Related Application

The present application is a continuation in part of United States Provisional Patent Application No. 60/024,404, filed August 22, 1996 and a continuation-in-part of United States Provisional Patent Application No. 60/028,169, filed October 11, 1996.

Technical Field of the Invention

The field of the present invention is HIV therapy. More particularly, the present invention pertains to a process of determining the efficacy or efficiency of drug treatment in HIV infected subjects by determining the level of HIV in monocytes of the subject.

Background of the Invention

HIV is known to productively infect a variety of different cell types *in vitro* and *in vivo*. The extent to which HIV infects and replicates in these cells has important implications concerning dissemination from portals of entry, cell function, and disease progression given the finite number of target cells. End products of viral replication including expression of unspliced HIV mRNA and plasma free virus has led to virologic determinants as a measure of disease state and therapeutic efficacy. A marked increase in the ratio of unspliced to spliced HIV mRNA as might occur during the shift from latent to productive infection precedes precipitous drops in CD4 count. Plasma viral load has been shown to correlate with disease progression and has been used to determine HIV kinetics *in vivo*. These measurements, however, fail to provide information on the cell type of origin–a weakness considering the effects of HIV expression on cell function, the role of infected cells in transmission and dissemination, and the therapeutic potential of blocking cell type specific co-receptors.

Plasma viral burden analysis have allowed researchers to estimate kinetic parameters of HIV-1 life cycle *in vivo*. The life span of productively infected T-lymphocytes was estimated to be 2.2 days. The

the subject are measured over the course of treatment with one or more drugs (e.g., AZT, 3TC, DDC). As drug treatment efficacy increases, the levels of HIV RNA in monocytes decreases. Conversely, where a subject develops resistance to a drug, that resistance is evident from an increase or lack of decrease in monocyte HIV RNA levels. In other words there is a direct correlation between the effectiveness of treatment and monocyte HIV RNA levels. Preferred monocytes for use in this invention are $CD14^+$ monocytes.

In accordance with the present invention, dual immunophenotyping PCR *in situ* hybridization (DIPDISH) is used to detect cells containing HIV-1 DNA, dual immunophenotyping fluorescence *in situ* hybridization (DIPFISH) is used to detect and quantify gag-pol mRNA in cells and quantitative RNA analysis is used to quantify plasma viral load. The present invention discloses that monocytes, and particularly $CD14^+$ monocytes, are persistently productive of HIV message. Furthermore, the levels of HIV mRNA in those monocytes respond in parallel with plasma viral load to drug therapy. As viral message production is an earlier event in virion production a process of the present invention is more a more sensitive indicator of drug efficacy and drug resistance than prior art methods.

Productively infected cell types in patients infected by HIV have been identified and quantified. As shown previously, very few CD4 positive lymphocytes were productively infected by HIV although many contain proviral DNA. The present invention discloses that monocytes are the major productively infected cell type in HIV seropositive individuals and viral production in these cells is altered by antiretroviral therapy. The percentage of productively infected monocytes corresponded with viral burden analysis in patients on no, single, combination, and triple drug therapy.

therapy. Forty HIV seropositive patients from the VA Lakeside Hospital followed routinely were evaluated using CD4 count, DIPDISH, DIPFISH, and quantitative RNA. Five HIV seronegative patients were evaluated using the same assays. Patients were either on no therapy, single drug therapy with AZT (200 mg three times/day) or DDC (0.75 mg three times/day) , combination therapy with AZT and 3TC (150 mg two times/day), or triple therapy with AZT 3TC and either Indinavir (800 mg three times/day) or Saquinavir (600 mg three times/day). All patients were on therapy for at least 30 days prior to blood donation. PBMCs were isolated from fresh heparinized blood layered on a Histopaque 1077 (Sigma, St. Louis, MO) discontinuous density gradient and centrifuged at 600xg for 30 minutes at ambient temperature. The turbid layer was removed, washed twice with 3 volumes of RPMI and once with phosphate buffered saline (PBS).

Detailed Description of the Invention

The present invention provides a process for determining the efficacy of anti-viral therapy in an HIV-infected subject receiving such therapy. The process includes the steps of a) detecting the level of transcriptionally active HIV in monocytes of the subject at a plurality of different times, b) comparing the detected HIV levels, and c) correlating changes in the detected HIV levels over time with the therapy. The process can be used to monitor the efficacy of treatment with any anti-HIV agent such as AZT, 3TC, DDc, Indivar, or Saquinavir. Decreases in HIV levels over time indicate an efficacious treatment. Increases in detected HIV levels over time indicate resistance to treatment.

The level of transcriptionally active HIV is detected by measuring the level of HIV mRNA and, preferably gag-pol HIV mRNA. The HIV mRNA is detected using *in situ* hybridization. *In situ* hybridization is accomplished by exposing monocytes *in situ* to an oligonucleotide probe that specifically hybridizes to at least of a portion of the HIV mRNA. The probe is labeled with a detectable marker, the detection of which indicates the presence of HIV mRNA.

(600 mg, three times a day). All patients were on therapy for at least 30 days prior to blood donation.

Histogram gates were set based on a negative control cocktail directed against cytomegalovirus and on a positive control cocktail directed against 28 ribosomal RNA. Fine tuning of the gates on each patient sample were set based on internal HIV-negative and CD4 or CD14 negative populations. $CD4^+$ T-lymphocytes containing HIV-1 DNA ranged from 4% to 43%. The percentage of cells containing HIV-1 DNA did not correlate with CD4 count or drug therapy suggesting that this population may be a reservoir for defective viral genomes, non-productive infection of resting T-cells, or provirus capable of subsequent activation.

Cells and cell lines. PBMCs were isolated from fresh heparinized blood layered on a Histopaque 1077 (Sigma, St. Louis, MO) discontinuous density gradient and centrifuged at 600 x g for 30 minutes at ambient temperature. The turbid layer was removed, washed twice with 3 volumes of RPMI and once with phosphate buffered saline (PBS). The ACH-2 cell line (AIDS Research and Reagent Program, NIAID, NIH, Bethesda, MD), containing a single copy of integrated HIV-1 proviral DNA per cell, was harvested at an early passage number and used as the HIV-1-infected cell copy number control.

Dual Immunophenotyping/Fluorescence in situ Hybridization (DIPFISH). To determine the cell types containing HIV RNA using DIPFISH, cells were labeled with optimized concentrations of phycoerythrin (PE)-conjugated antibodies specific for the cell types of interest (CD4, CD14) and fixed and permeabilized by the addition of 50 µl of a water-soluble, non-aldehyde fixative, Permeafix (Ortho Diagnostics, Inc.) per 10^6 cells at ambient incubation temperature for at least 60 min. The cells were then washed twice in PBS, pH 7.4 at ambient temperature and once in 2 x SSC at ambient temperature.

allowed to hybridize with the respective oligonucleotide probe at 56°C for 2 hours.

After hybridization, the cells were washed for 30 minutes with 2x SSC/50% formamide/500 µg/ml bovine serum albumin (BSA) at 42°C, 30 minutes with 1x SSC/50% formamide/500 µg/ml BSA at 42°C, 30 minutes with 1x SSC/500 µg/ml BSA at ambient temperature and then briefly with PBS at ambient temperature. Following the last wash, the cells were resuspended in 80µl of PBS and 20µl strepavidin-phycoerythrin (PE) and incubated for 30 minutes at ambient temperature. The cells were then washed in PBS as described above.

Flow Cytometry. The cell suspension was filtered through a 37 mm nylon mesh and analyzed by flow cytometry using an EPICS XL flow cytometer. Laser excitation was 15 mW at 488 nm, and the FITC and PE florescence was detected with standard optical filter set-up (550 dichroic, 525 bandpass (FITC) and 585 bandpass (PE)). Instrument sensitivity was standardized before each experiment employing Immuno-Bright calibration beads (Coulter Source, Marriette, GA). The percent fluorescence-positive cells was determined by integration over a range of 0.2% positive counts on the identically treated negative sample (100% uninfected PBMCs).

Viral Burden. Quantitative RNA determinations were performed on plasma using the Amplicor RNA kit (Roche Molecular Systems, Alameda, CA) as per manufacturer instructions.

In peripheral blood, a significant proportion of PBMCs contain HIV-1 DNA while very few contain transcriptionally active virus. Simultaneous immunophenotyping not only allowed unequivocal identification of infected cell subtypes but also enriched cell subtypes for the various infected cell types present in the blood. To determine the cell types with productive or latent HIV infection, immunophenotyping was combined with a novel *in situ* hybridization strategy using 220 5'- and 3', 6-carboxyfluorescein-labeled oligonucleotides complementary to gag-pol targets or with *in situ* PCR

monocytes expressing HIV-1 mRNA may represent production of free virus predominantly in other cell types as may occur in the phenotypic switch preceding symptomatic infection. Even in the extreme cases of a patient with a high viral titer in spite of triple therapy or a patient with a low viral titer in the absence of therapy, the percentage of infected monocytes paralleled the viral load.

Nine out of fifteen patient (60.0%) with a high percentage of monocytes expressing HIV-1 mRNA had viral loads over 50,000 copies/ml while four out of twenty-three patient (17%) with less than 50% monocytes expressing HIV-1 mRNA had viral loads over 50,000 copies/ml. As viral message production is an earlier event in the viral lifecycle, this measurement is likely a more sensitive indicator of drug efficacy and drug resistance.

Productively infected cell types in blood from patients infected by HIV were identified and quantified. CD14 positive monocytes were the major persistently productive cells infected with HIV. A specific subtype of infected monocytes was not identified even though CD14 low, CD16 high cells phenotypically resembling macrophages have been identified in the peripheral blood of HIV seropositive individuals. Interestingly, productively infected monocytes in several patients exhibited an increased CD14 mean peak fluorescence relative to the HIV negative monocytes in the same sample. Increases in CD14 expression in monocytes has been shown in the peripheral blood of HIV-infected patients although the presence of HIV in these cells was not determined in this previous study. CD14 is a glycosyl-phosphatidynositol -anchored molecule on the surface of monocytes and to a lesser extent granulocytes. Monocyte activation by lipopolysaccharide requires CD14, therefore alterations of CD14 could conceivably explain the functional defect of monocytes at the cellular level in HIV-infected individuals. As shown previously, very few CD4 positive lymphocytes were productively infected by HIV although many contain proviral DNA.

Quantification of infected monocytes/macrophages is not only critical for a complete kinetic model of HIV infection but also for therapeutic monitoring. Since these cells are not destroyed by viral production and persistently infected cells such as macrophages, may have a lifespan five to six times longer than infected T-lymphocytes, these cells are ideal for monitoring HIV activity at the cellular level including lymphoid tissue such as tonsil and lymph nodes.

Clearly, the contribution of productively-infected monocytes to the free virus pool must be distinctly defined. With the discovery of new tropism dependent co-receptors for HIV and inevitable therapy directed at blocking these co-receptors, determination of productively infected cell types and their contribution to the free virus pool is critical. The ease of the technologies used to define the viral lifecycle in this study most likely portends the end of surrogate markers as we know them and reaffirms the trend toward viral lifecycle monitoring.

10. The process of claim 9 wherein the fluorescent label is a 5- or 6- carboxyfluorescein.

11. The process of claim 2 wherein the CD14+ monocytes are labeled with an antibody against CD14, which antibody is labeled with a detectable marker.

12. The process of claim 11 wherein the detectable label on the antibody is a fluorescent label.

13. The process of claim 12 wherein the detectable fluorescent label is phycoerythrin.

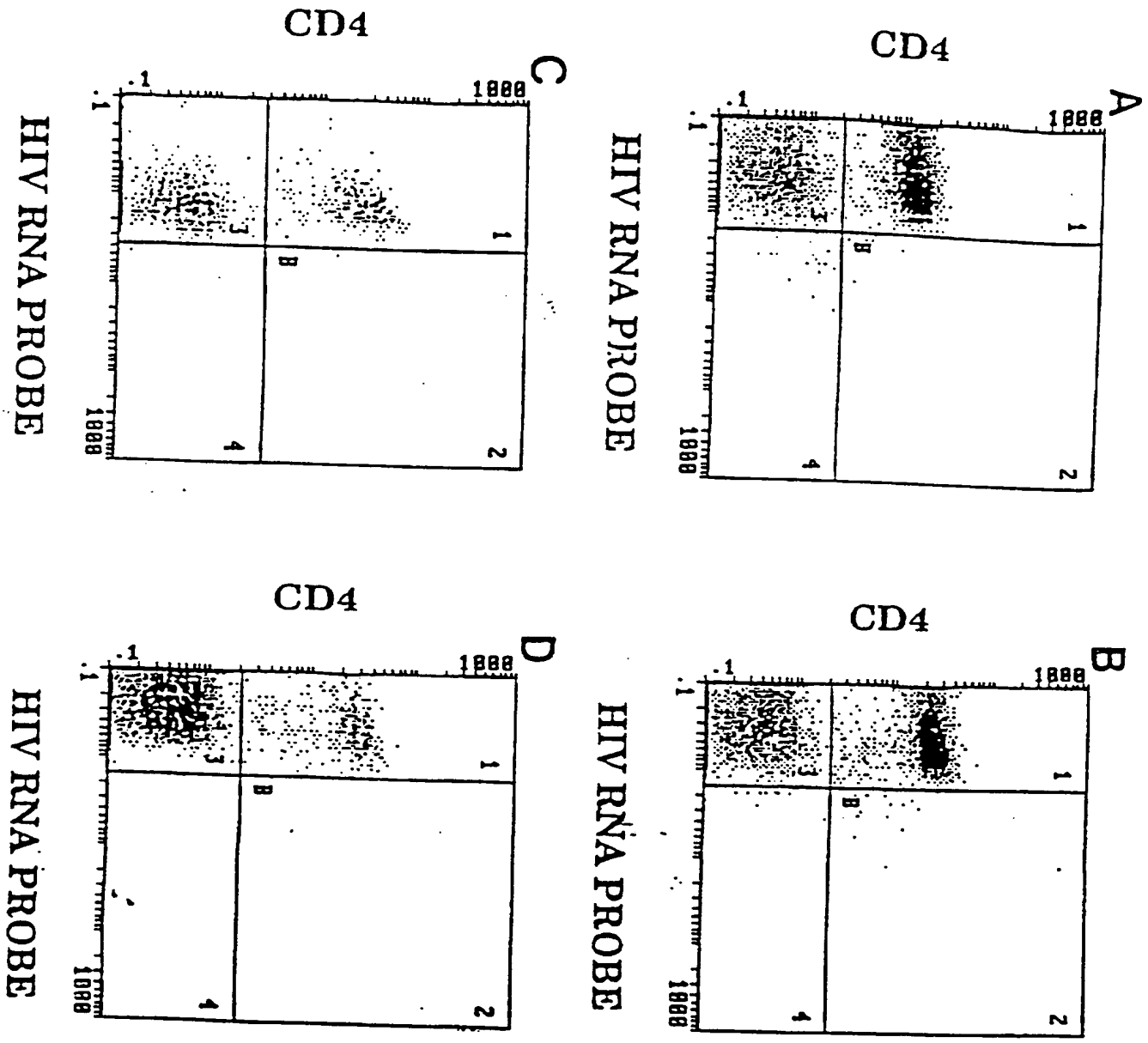

FIG. 2a

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/14870

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 23574 A (KOZAL MICHAEL J ;MERIGAN THOMAS C (US); KATZENSTEIN DAVID A (US);) 25 November 1993 see the whole document ---	1
A	WO 96 14437 A (AKZO NOBEL NV ;ROMANO JOSEPH W (US); PAL RANAJIT (US)) 17 May 1996 see the whole document ---	1
A	LEWIS D E ET AL: "DETECTION AND SIGNIFICANCE OF HIV SEQUENCES IN HIV INFECTION" PROGRESS IN MEDICAL VIROLOGY, vol. 40, 1 January 1993, pages 19-47, XP002000193 see page 39; table 2 ---	1-13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1997	30/12/1997

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/14870

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9323574 A	25-11-93	AU 4248593 A	13-12-93
		US 5631128 A	20-05-97
		US 5650268 A	22-07-97
WO 9614437 A	17-05-96	US 5660979 A	26-08-97
		AU 4160196 A	31-05-96
		CA 2204372 A	17-05-96
		EP 0791079 A	27-08-97
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US 5543303 A	06-08-96	NONE	